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Proteolytic activity of recombinant DegP from *Chromohalobacter salexigens* BKL5Dewi Fitriani^a, M. Saifur Rohman^{a,b,*}, Irfan D. Prijambada^{a,b}^a Graduate Program in Biotechnology, Universitas Gadjah Mada, Yogyakarta, Indonesia^b Laboratory of Agricultural Microbiology, Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Indonesia

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ABSTRACT

Background: DegP is a serine protease that specifically cleaves and refolds unfolding proteins in the periplasmic space of the cells. To date, there is no information regarding DegP from halophilic bacteria. *Chromohalobacter salexigens* BKL5 is a moderately halophilic bacterium that has the ability to grow in a media containing more than 15% salt. Therefore, the objectives of this work were to clone and overexpress DegP-encoding gene from *C. salexigens* BKL5 and characterize its biochemical properties.

Results: DegP-encoding gene was overexpressed in *Escherichia coli* BL21(DE3) CodonPlus in an active form. SDS-PAGE analysis showed that the molecular weight of the recombinant DegP was 45 kDa. Size-exclusion chromatography analysis suggested that recombinant DegP was present in two multimeric states, hexameric and dodecameric, with molecular weights of 297.9 and 579.12 kDa, respectively. Both conformations were enzymatically active when casein was used as substrate for enzymatic assay. Circular dichroism analysis showed that recombinant DegP was composed of 0.21–0.29 helical content, which was comparable to the helical content in the crystal structure of *E. coli* DegP. The basic/acidic residue ratio of recombinant DegP was 0.56, which was slightly higher than that of DegP from extreme halophiles (average, 0.45) but significantly lower than that of DegP from nonhalophiles (average, 0.94).

Conclusions: Recombinant DegP from *C. salexigens* BKL5 showed proteolytic activity when β -casein was used as a substrate. *In silico* analysis indicated that recombinant DegP had characteristics similar to those of halophilic proteins depending on its amino acid composition.

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1. Introduction

DegP is a protein that exhibits ATP-independent protease activity and plays an important role in the removal of toxic protein misfolding in the periplasmic space of the cell [1]. The protein was first detected in *Escherichia coli* and recognized as protease Do [2,3,4,5,6]. Currently, DegP is known to be present in all the kingdoms of life, although its existence in cells is not required [7]. DegP belongs to the high-temperature requirement A (HtrA) protein family, the enzymatic activity of which is upregulated by various environmental stresses, such as heat shock, oxidative stress, and the presence of reducing agents [8,9]. In *E. coli*, DegP is upregulated by both the Cpx and σ^E protein quality control pathways under conditions of protein folding stress [10,11]. In addition, DegP can act as a chaperon to assist protein folding in the periplasm [12].

The most extensively studied DegP is from *E. coli* [13,14]. The monomeric structure of *E. coli* DegP consists of a protease domain and two PDZ (*Postsynaptic density proteins*, *Drosophila disc large tumor suppressor*, *Zonula occludens-1 protein*) domains [15]. Crystal structure analysis of *E. coli* DegP shows that the protein has a hexameric structure that is composed of two trimeric forms [13]. However, the orientation of catalytic triad residues in the hexameric conformation is completely distorted, rendering it enzymatically inactive. Further analysis of larger structures of DegP, *i.e.*, the 12-mer and 24-mer conformations, shows that the catalytic triad residues orient to catalytically act upon substrate binding. In addition, the activity of the larger structures is induced by protein-substrate interaction [16].

Halophilic bacteria can adapt to environments with high salt concentrations [17,18,19,20]. To adapt to such environments, halophilic bacteria frequently accumulate compatible solutes (such as highly water-soluble sugar, alcohols, amino acids, betaines, and ectoines or their derivatives) to protect their cellular components from the destructive effect of high salt concentrations [20,21].

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However, high salt concentrations may affect the folding mechanism of periplasmic space proteins in halophilic bacterial cells.

Because of their living conditions, it is very interesting to study the structure and function of DegP proteins in halophilic bacteria. There are no reports so far on whether the DegP from halophilic bacteria has a conformation similar to that of *E. coli* DegP. Therefore, the objective of this work was to clone and overexpress the DegP-encoding gene (*degP*) from *Chromohalobacter salexigens* BKL5, a halophilic bacterium that was previously isolated by Bledug Kuwu in our laboratory. *C. salexigens* BKL5 has an ability to grow in media containing 15%, 20%, and 25% salt at maximum specific growth rates (μ) of 1.4×10^{-2} , 1.1×10^{-2} , and $1.0 \times 10^{-3} \text{ h}^{-1}$, respectively. The results indicate that the growth rate of *C. salexigens* BKL5 significantly decreased at salt concentrations 20% or higher [22]. Most moderately halophilic bacteria are unable to grow at concentrations higher than 10% salt. Therefore, *C. salexigens* BKL5 is a good model for studying the structure and function relationship of DegP in halophilic bacteria.

2. Materials and methods

2.1. Genomic DNA extraction and amplification of DegP gene

Previously, *C. salexigens* BKL5 was cultured in artificial seawater medium containing (% w/v) NaCl 15.6, MgCl₂ 1.3, MgSO₄ 0.7, H₂O 2.0, CaCl₂ 0.6, H₂O 0.1, KCl 0.4, NaHCO₃ 0.02, NaBr 0.05, and yeast extract 1, at room temperature for 16 h. Cell pellets were then harvested by centrifugation, and genomic DNA was extracted using a Wizard Genomic DNA purification kit (Promega). This genomic DNA was then used as a template for the amplification of the DegP-encoding gene. The forward and reverse primers were as follows: *degP*-for: TATACATATGCGCGACCTGCCGACTTCAC and *degP*-rev: ATAGAAT TCTACTCTGCTTTTCGGTCTC. The forward and reverse primers were designed according to the *degP* gene of *C. salexigens* DSM3043 (GenBank Accession No. CP000285.1:1849594-1850952). In these sequences, the underlined bases show the position of *NdeI* (5'-primer) and *EcoRI* (3'-primer) sites. Polymerase chain reaction was performed with a Takara Thermal Cycler Dice Mini TP-100 (Takara) using KODPlus Neo (Toyobo) according to the manufacturer's instructions. DNA oligomers for PCR were synthesized by Hokkaido System Science (Sapporo, Japan). The DNA sequence was confirmed by sending the PCR product to the 1stBASE company, Malaysia.

2.2. Plasmid construction

A pET plasmid was used for the overexpression of DegP. The plasmid was constructed by ligating the DegP-encoding gene fragment (*degP*) into the *NdeI*-*EcoRI* site of pET25b. The resultant plasmid (pET-*degP*) was then used to transform *E. coli* DH5 α . *E. coli* DH5 α harboring pET-*degP* was then grown on Luria-Bertani agar medium supplemented with 100 $\mu\text{g/ml}$ of ampicillin. Positive cloning was checked by the colony PCR method. The positive clone was then grown in Luria-Bertani broth medium supplemented with 100 $\mu\text{g/ml}$ ampicillin for plasmid extraction.

2.3. Overexpression and solubility check of recombinant DegP

For the overexpression of the DegP gene, *E. coli* BL21(DE3) CodonPlus was transformed with pET-*degP* and grown in Luria-Bertani medium supplemented with 100 $\mu\text{g/ml}$ ampicillin and 25 $\mu\text{g/ml}$ chloramphenicol. The culture was incubated at 37°C. When an A₆₀₀ of approximately 0.5 was reached, 1 mM of isopropylthio- β -D-galactopyranoside (IPTG) was added. The culture was then incubated for an additional 3 h. After that, the culture was harvested by centrifugation at 6000 \times g for 10 min. A cell pellet was then washed using 10 mM Tris-HCl (pH 8.0). The cell pellet was then diluted with 2 \times SDS-PAGE loading buffer and boiled for 5 min. The

cell pellet was then subjected to SDS-PAGE analysis on 15% polyacrylamide gel, followed by staining with Coomassie Brilliant Blue (CBB). For a solubility check of the expressed recombinant DegP, 100 μl of cell culture was harvested by centrifugation, and the cell pellet was collected. The cell pellet was disrupted by sonication and then centrifuged at 15,000 \times g for 30 min. The pellet and supernatant were separated. The pellet was washed with 10 mM Tris-HCl pH 8.0. The supernatant and pellet were then dissolved with 50 and 100 μl of the 2 \times SDS-PAGE loading buffer, respectively. The solubility was checked on 15% SDS-PAGE, followed by staining with CBB.

2.4. Overproduction of recombinant DegP

For the overproduction of recombinant DegP, *E. coli* BL21(DE3) CodonPlus containing pET-*degP* was grown in 500 ml Luria-Bertani medium supplemented with 100 $\mu\text{g/ml}$ ampicillin and 25 $\mu\text{g/ml}$ chloramphenicol. When the A₆₀₀ reached 0.5, 1 mM IPTG was added, and the cultivation was continued for an additional 3 h. Cells were harvested by centrifugation at 6000 \times g for 10 min, suspended in 10 mM Tris-HCl (pH 8.0), and disrupted by French press. Then the cells were centrifuged at 30,000 \times g for 30 min. The supernatant was collected and applied to a HitrapQ column (5 ml, GE Healthcare) equilibrated using the same buffer. The protein was eluted from the column using a linear gradient of NaCl from 0 to 1 M NaCl in 10 mM Tris-HCl (pH 8.0). The protein-containing fractions were collected, dialyzed against 20 mM Tris-HCl (pH 8.0), and concentrated to 1 ml of concentrated protein using Amicon Ultra 0.5 ml (Millipore). The concentrated protein was then applied to HiLoad 16/60 Superdex 200 pg column (GE Healthcare) equilibrated using the same buffer. The flow rate was set at 0.5 ml/min. The protein-containing fractions were collected and used for further analyses. The molecular mass of protein was estimated by gel filtration column chromatography using bovine serum albumin (BSA) (67 kDa), ovalbumin (44 kDa), chymotrypsinogen A (25 kDa), and RNase A (14 kDa) as standard proteins. The purity of protein was analyzed by SDS-PAGE on a 15% polyacrylamide gel [23], followed by staining with CBB. The protein concentration was determined from UV absorption using an A₂₈₀ value of 0.646 for a 0.1% solution. The value was calculated using an ϵ of 1576 M⁻¹ cm⁻¹ for Tyr and 5335 M⁻¹ cm⁻¹ for Trp at 280 nm.

2.5. Circular dichroism

Circular dichroism (CD) spectrum was measured on a J-725 spectropolarimeter (Japan Spectroscopic) at 20°C. The far UV CD spectra were obtained using a solution containing 0.1 mg/ml protein in 20 mM Tris-HCl (pH 8.0) in a cell with an optical path length of 2 mm. The mean residue ellipticity, θ , which has the units of deg cm² dmol⁻¹, was calculated by using an average amino acid molecular weight of 110.

2.6. Enzymatic activity

Recombinant DegP activity was determined using 30 μg of casein substrate in 50 mM Tris-HCl (pH 7.5) supplemented with 100 mM NaCl. The reaction mixture was incubated at 30°C. Incubation was terminated at a specified time by the addition of chloroform to the reaction mixture and then incubation on ice for 30 min. The mixture was centrifuged at 30,000 \times g for 10 min. The pellet was then dried and dissolved using protein loading buffer. The cleavage product was then analyzed using SDS-PAGE on 15% polyacrylamide gel, followed by staining with CBB.

2.7. In silico analyses of *C. salexigens* BKL5 DegP

The amino acid sequence of DegP was deduced from the nucleic acid sequence of the DegP-encoding gene (*degP*). The sequence of *degP* was

assembled using DNA Baser Sequence Assembler ver. 3.5 and then analyzed using BLASTx. The *degP* gene sequence was deposited in GenBank under accession no. MF071199. The composition and physicochemical properties of the deduced amino acid sequence were then analyzed using ExPASy ProtParam (<http://web.expasy.org/cgi-bin/protparam/protparam>) [24].

3. Results and discussion

3.1. Cloning and expression of recombinant *C. salexigens* BKL5 DegP

As the DegP protein contains a signal peptide that is useful for protein translocation to cell membranes, in this work, we eliminated the signal peptide of DegP by amplifying the mature domain of the DegP protein. Therefore, the expressed recombinant protein will accumulate intracellularly instead of translocating to cell membranes. From the PCR results, the amplified DegP gene was 1372 bp long, and it would theoretically produce a recombinant DegP protein with a molecular weight of 50 kDa. Amino acid sequence analysis indicated that recombinant *C. salexigens* BKL5 DegP also consists of a serine protease domain and two PDZ domains, which is similar to *E. coli* DegP sequence (data not shown).

The PCR product was then cloned into the pET system to produce the pET-*degP* recombinant plasmid (Fig. 1). The recombinant plasmid was then overexpressed in *E. coli* BL21(DE3) CodonPlus. Overexpression was verified by SDS-PAGE: a sharp and distinct band was observed at the 45 kDa position (Fig. 2). This value was comparable to the estimated size calculated from the length of the DNA fragment.

The overexpressed protein was accumulated in the cell in abundance and presented in a soluble form (data not shown). This result indicated that the expressed protein was present in an active form. However, DegP is a serine protease that exhibits enzymatic activity in multimeric form, which could not be shown by the denaturing SDS-PAGE gel analysis. It needs to be confirmed whether the recombinant protein was overexpressed in the multimeric form. Therefore, gel filtration analysis was employed to analyze the multimeric state of recombinant DegP.

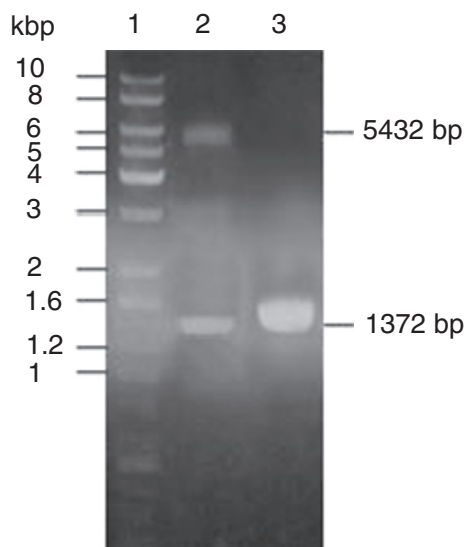


Fig. 1. Agarose gel electrophoresis of the recombinant DegP gene from *C. salexigens* BKL5 cloned in a pET system. Electrophoresis was conducted on 0.8% agarose gel. KAPA Universal DNA ladder (lane 1); pET-*degP* (lane 2); PCR product of *degP* (lane 3).

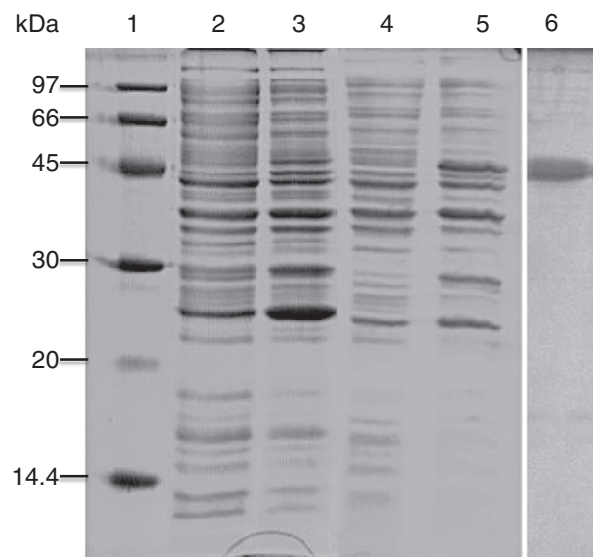


Fig. 2. SDS-PAGE of recombinant DegP overproduced in *E. coli* BL21(DE3) CodonPlus. Samples were subjected to 15% SDS-PAGE and stained with Coomassie Brilliant Blue. Low-molecular-weight marker kit (GE Healthcare) (lane 1); whole cell extract of the cell harboring pET system before and after induction (lanes 2 and 3, respectively); whole cell extract of the cell harboring pET-*degP* before and after induction (lanes 4 and 5, respectively); purified recombinant DegP (lane 6).

3.2. Overproduction, purification, and multimeric state analysis of recombinant DegP

To examine of the multimeric state of recombinant DegP, the recombinant protein was overproduced and purified; the yield of the recombinant protein DegP was 2 mg/l culture. The purified protein was then subjected to size-exclusion chromatography. Gel filtration analysis indicated that there were two main peaks (Fig. 3a). After collecting the fraction of each major peak, SDS-PAGE analysis was employed. SDS-PAGE analysis indicated that recombinant DegP was present in both major peaks (Fig. 3b). Molecular weight calculations indicated that recombinant DegP was present in two multimeric states with molecular weights of 579.12 kDa (peak I) and 297.9 kDa (peak II) (Table 1). The results showed that recombinant DegP was present in dodecameric (12 subunits) and hexameric (6 subunits) forms. Multi-subunit structure analysis indicated that DegP of *C. salexigens* BKL5 was similar to *E. coli* DegP.

Secondary structure prediction based on the far UV CD spectrum (200–250 nm) (Fig. 4) showed that recombinant DegP was composed of 0.21–0.29 helical content. That is, recombinant DegP of *C. salexigens* BKL5 contains only 21–29% helical structures. This result was comparable to the helical content of *E. coli* DegP (24%, as calculated from a monomeric DegP crystal structure). This result suggested that the overall structure of *C. salexigens* BKL5 DegP was similar to that of *E. coli* DegP.

3.3. Enzymatic activity of recombinant *C. salexigens* BKL5 DegP

The enzymatic activity of recombinant DegP was determined using casein as a substrate. The casein that was used as substrate was composed of α -, β -, and γ -casein. The enzymatic activity assay showed that recombinant DegP had cleaved most of the β -casein substrate after 60 min of incubation (Fig. 5). These results indicated that both the dodecameric and hexameric forms of recombinant DegP from *C. salexigens* BKL5 were functional, mainly for their protease activity. This result was intriguingly different from that of the *E. coli* DegP crystal structure reported by Krojer et al. [14]. On the basis of the *E. coli* DegP crystal structure, the hexameric state of DegP was

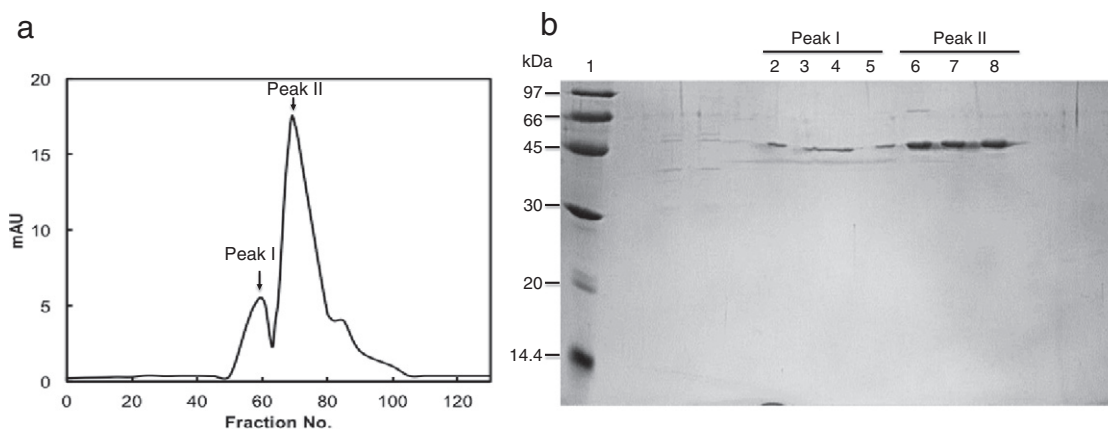


Fig. 3. Size-exclusion chromatography analysis of recombinant *C. salexigens* BKL5 DegP. The recombinant DegP eluted from HitrapQ column was applied to a HiLoad 16/60 Superdex 200 pg column equilibrated using 20 mM Tris-HCl pH 8. (a) Chromatogram of recombinant DegP and (b) SDS-PAGE analysis of recombinant DegP, which was present in both fractions (peaks I and II). Low-molecular-weight marker kit (GE Healthcare) (lane 1); fractions eluted from peak I (lanes 2–5); fractions eluted from peak II (lanes 6–8).

believed to be its resting state. Therefore, in its hexameric state, DegP should be in an inactive form. Our results clearly showed that the hexameric state of *C. salexigens* BKL5 DegP was very active as a protease at 30°C. It was previously reported that DegP could switch its function from chaperon to protease when the temperature increased above 28°C. In temperatures below 28°C, the Ser-210 residue was in an inactive conformation, but following an increase in temperature, Ser-210 changed into an active conformation [12,16]. The crystallization of hexameric *E. coli* DegP was performed at 18°C; therefore, it was not surprising that the hexameric state indicated an inactive form. In other words, the hexameric form will oligomerize into larger structures when DegP meets its substrate.

3.4. *In silico* analyses of recombinant DegP

To check whether recombinant DegP of *C. salexigens* BKL5 had the characteristics of halophilic proteins, these characteristics were analyzed *in silico*. The *in silico* analyses were performed using ProtParam (<http://web.expasy.org/protparam/>) [24]. The parameter used for the analysis was the ratio of basic to acidic amino acid residues in the protein [17,25]. In this work, we compared the ratio of basic to acidic amino acid residues of *C. salexigens* BKL5 DegP to those of DegP from nonhalophilic bacteria and extremely halophilic archeon (Table 2).

The amino acid composition of recombinant *C. salexigens* BKL5 DegP was dominated by negatively charged residues, with a basic to acidic residue ratio of 0.56. This value was slightly higher than that of DegP from extremely halophilic bacteria (average, 0.42) but significantly lower than that of DegP from nonhalophiles (average, 0.94). These results indicate that DegP from *C. salexigens* BKL5 belongs to the group of halophilic proteins on the basis of its amino acid composition. Although most moderate halophilic bacteria accumulate compatible solutes to protect their cytoplasmic proteins, the extracellular and membrane-bound proteins of halophilic bacteria are expected to display halophilic protein characteristics. Because DegP is usually

present in the periplasmic space, it is reasonable that recombinant *C. salexigens* BKL5 DegP had characteristics similar to halophilic proteins.

4. Conclusion

In this work, we successfully cloned and overexpressed recombinant DegP from the halophilic bacterium *C. salexigens* BKL5. Recombinant DegP showed proteolytic activity when β -casein was used as a substrate. *In silico* analysis indicated that recombinant DegP had characteristics similar to those of halophilic proteins based on its amino acid composition.

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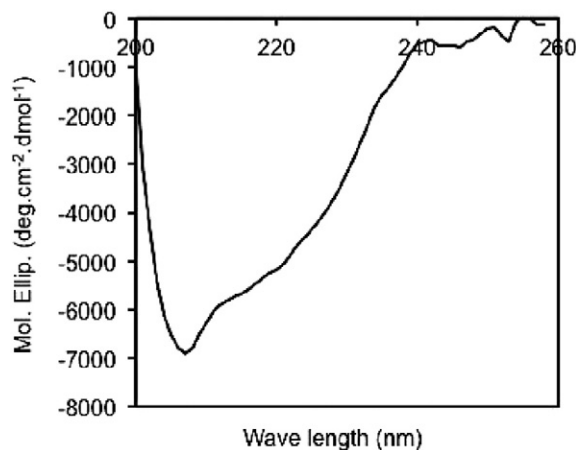


Fig. 4. Far UV CD spectrum of recombinant DegP. This spectrum was obtained at pH 8 and 20°C as described in Materials and Methods.

Table 1

Comparison of recombinant DegP molecular weight obtained from theoretical calculations and gel filtration analysis. The result was used to predict the multimeric state of each eluted recombinant DegP from the gel filtration column.

Peak	Molecular weight (kDa)		Multimeric state
	Calculated	Gel filtration	
I	574.8	579.12	Dodecamer
II	287.4	297.9	Hexamer

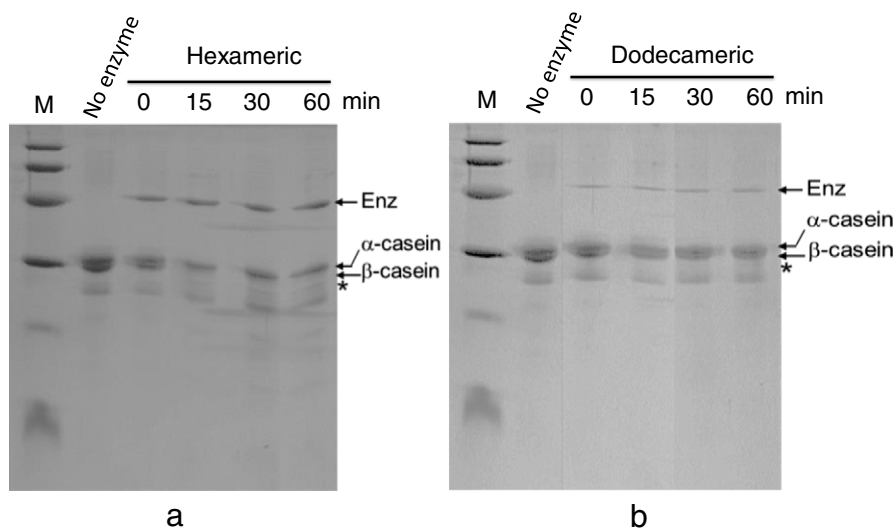


Fig. 5. Enzymatic activity assay of recombinant DegP. The enzymatic activity assay was performed using casein as the substrate as described in Materials and Methods. The proteolytic specificity of both multimeric states against β -casein are shown. The faint cleavage product is shown by an asterisk (*).

Table 2

Comparison of the ratio of basic to acidic amino acid residues in recombinant DegP with nonhalophilic and extremely halophilic DegP.

Species	Number of amino acid residues		Ratio basic/acidic	Identity (%)	Accession no.
	Basic (Lys, Arg)	Acid (Asp, Glu)			
<i>Halobacterium jilantaiense</i>	16	43	0.37	37	SEW25498.1
<i>Habacterium sp. DL1</i>	15	43	0.35	35	WP_009487642.1
<i>Halobacterium CBA1132</i>	19	51	0.37	35	WP_058983923.1
<i>Halobacterium hubeiens</i>	18	50	0.36	34	WP_059057431.1
<i>Halobacterium salinarum</i>	25	38	0.66	35	WP_010902123.1
Average			0.42		
<i>Chromohalobacter israelensis</i>	37	69	0.54	86	WP_035412532.1
<i>Chromohalobacter salexigens</i>	42	75	0.56	86	WP_011506927.1
<i>Halomonas lutea</i>	37	57	0.65	62	WP_019018688.1
<i>Halomonas xianhensis</i>	41	66	0.62	63	SFH79220.1
<i>Halomonas smymensis</i>	39	70	0.56	62	WP_016854321.1
Average			0.58		
<i>Pseudomonas stutzeri</i>	45	55	0.82	53	WP_014819520.1
<i>Pseudomonas fluorescens</i>	49	51	0.96	52	WP_078741282.1
<i>Escherichia coli</i>	41	38	1.08	38	WP_001467943.1
<i>Bacillus cereus</i>	46	49	0.94	36	WP_002066653.1
<i>Streptococcus pneumoniae</i>	46	51	0.90	53	CJL10589.1
Average			0.94		
<i>Chromohalobacter salexigens BKL5</i>	37	66	0.56	100	MF071199

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